IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Attorney Docket No. 006420.00003)

In the Application of:	)	
	)	
Arora, et al.	)	
	)	Examiner: Clark, Amy L.
Serial No.: 10/525,992	)	
	)	Group Art Unit: 1655
Filing Date: February 28, 2005	)	
	)	
For: Herbal Extract Comprising A Mixture of Saponins Obtained From Sapindus Trifoliatus for Anticonvulsant Activity	)	

DECLARATION OF SUDERSHAN K. ARORA UNDER 37 C.F.R. 1.132

1. I, Sudershan K. Arora, declare as follows.

2. I am an inventor named in the present application. My present title is President – Novel Drug Discovery & Development of Lupin Ltd. My curriculum vitae is attached as Exhibit 1 hereto.

3. I am submitting this Declaration in response to the Office Action mailed August 9, 2006 in the subject patent application.

4. Table 1 below is a redacted excerpt from the specification of the present patent application (page 19).

Table 1: Disclosure in US 10/525,992

S.No.	Receptor	Percent inhibition with *Sapindus trifoliatus*	
		2.5 µg/ml	**250 µg/ml**
1	GABA A, agonist Site	50.92	102.40
2	Glutamate AMPA Site	5.43	87.36
3	Glutamate Kainate Site	-15.70	87.29
4	Glutamate NMDA agonist Site	7.27	98.14
5	Glutamate NMDA glycine (Strychnine insensitive) site	14.50	85.33
6	GABA chloride TBOB	-5.12	85.03
7	Glutamate chloride	-2.72	89.49
8	Sodium site-2	19.98	69.54

5. A criterion of 50% inhibition or greater may be used to qualify a compound as active in binding experiments. *See* Exhibit 5 to this Declaration (literature specification from Novascreen).

6. As shown in Table 1, at 250 µ/ml, *Sapindus trifoliatus* is qualified as active under the Novascreen criterion.

7. *Emblica officinalis* was tested for binding towards the sites listed in the above table. Receptor binding assays were conducted at Novascreen, U.S.A. The results are summarized in Table 2 hereinbelow (See Exhibit 4).

Table 2: Receptor Binding affinity with the extract of *Emblica officinalis*

S.No.	Receptor	Percent Inhibition with *Emblica officinalis*	
		2.5 µg/ml	**250 µg/ml**
1	**GABA A agonist Site**	**64.09**	**102.22**
2	**Glutamate AMPA Site**	-10.07	**56.20**
3	Glutamate Kainate Site	-1.12	39.30
4	**Glutamate NMDA agonist Site**	14.90	**71.82**
5	Glutamate, NMDA glycine (Strychnine insensitive) site	0.48	28.21
6	GABA chloride TBOB	-1.95	-0.03
7	Glutamate chloride	28.28	49.24
8	Sodium site-2	7.11	3.93

8. As seen, *Emblica officinalis* at 250 µg/ml fails the Novascreen criterion for Glutamate Kainate site, Glutamate, NMDA glycine (Strychnine insensitive) site, GABA chloride, TBOB, Glutamate chloride, and Sodium site-2.

9. An antimigraine formation prepared as per Gupta et al. was also tested for binding towards the foregoing sites (see Exhibit 4). The results are summarized in Table 3.

Table 3: Receptor Binding affinity with the antimigraine formulation mentioned in Gupta et al patent.

S.No.	Receptor	Percent inhibition (Gupta et al composition)	
		2.5 µg/ml	**250 µg/ml**
1	GABA A, Agonist Site	19.21	**95.95**
2	Glutamate, AMPA Site	-0.89	41.69
3	Glutamate, kainate Site	-1.16	25.68
4	Glutamate, NMDA agonist Site	15.26	**66.02**
5	Glutamate, NMDA glycine (Strychnine insensitive) site	4.03	42.60
6	GABA chloride,TBOB	-14.60	-3.77
7	Glutamate chloride	1.95	**84.35**
8	Sodium site 2	13.37	3.30

10. Gupta et al. showed markedly reduced binding relative to the inactive composition. Gupta et al. satisfied the 50% inhibition criterion only for three sub-types of receptors, and failed to satisfy the 50% inhibition criterion for Glutamate AMPA site, Glutamate Kainate site, Glutamate NMDA glycine (Strychnine insensitive) site, GABA chloride, TBOB, and Sodium site 2.

11. The saponin content of *Sapindus trifoliatus* and *Emblica officinalis* were evaluated via high performance liquid chromatography, conducted according to the following protocol:

Quantitative Analysis of Raw Material by High Performance Liquid Chromatography (HPLC)

Preparation of the sample: About 1g of the fruit pericarp powder was accurately weighed into a 250ml round bottom flask (RB Flask), 100ml water was added to the same and the total weight of the flask was noted. The content of the flask was then refluxed for 2hrs at 100°C. After cooling the flask was weighed again and water was added to adjust the lost volume to make up the solution to 250ml. From this sample solution, 20ml was transferred to a 100ml RB Flask and 5ml of 50% methanolic-HCl was added to it. The solution was then refluxed at 100 ^{0}C for 2hrs and then evaporated to dryness. The residue was dissolved in 10ml of diluent (A mixture of THF and Methanol in the ratio 30:70 v/v) and carefully transferred to 50ml volume. The samples were analysed and the saponin content were estimated as hederagenin by a quantitative HPLC method similar to that described hereinafter.

12. The following results were observed:

1. Total saponin content of *Sapindus trifoliatus* pericarp was analyzed as hederagenin and was found to be 2.65% w/w.

2. In case of *Emblica officinalis* fruit, hederagenin content was below the detection limit.

3. In the mixture of *Sapindus trifoliatus* and *Emblica officinalis* (1:1) hederagenin content was 1.01% w/w.

13. These results demonstrate that any hederagenin in the mixture of Gupta originated from the extract of *Sapindus trifoliatus* pericarp and that the presence of extract of *Emblica officinalis* deters the amount of hederagenin reducing the total content which is surprising.

14. For further verification, thin layer chromatographic analysis was performed. The analysis was performed according to the following protocol.

Thin Layer Chromatography (TLC) Materials and methods

A. Sample Preparation for the finger printing by TLC of 1% nasal spray: 1.5 mL of the sample was taken in a 10 mL volumetric flask and the volume was made up to mark with water. The content was carefully transferred to a 50 mL-separating funnel and extracted with 5 mL of n-Butanol 3 times. The organic layer was collected. The combined extract was transferred to a 50 mL round bottom flask and evaporated to dryness. The residue thus obtained was dissolved in about 2 mL methanol

B. Sample Preparation for Raw material: Around 1.0 gm of sample was taken in a 250 mL round bottom flask. 100 mL of water was added and refluxed at 100 ^{0}C for 2 hours. 10mL of extract was carefully transferred to a 50mL-separating funnel and extracted with 5 mL of n-Butanol 3 times. The organic layer was collected. The combined extract was

transferred to a 50mL round bottom flask and evaporated to dryness. The residue thus obtained was dissolved in about 2mL methanol.

C. Mobile Phase: Ethyl Acetate : Methanol : Water (80:10:10)

D. Detection: TLC plate is sprayed with 10% acqueous sulfuric acid, charred by heating and observed visually.

Details Of samples spotted on The TLC plate:

Raw Material:

Spot No	Details	Sample ID
1	*Sapindus trifoliatus*	SKJ-20-181-1
7	(1:1) Mix of *Sapindus trifoliatus* and *Emblica officinalis*	SKJ-20-181-5
13	Emblica officinalis	SKJ-20-181-3

Compositions:

Spot No	Details	Sample ID
2	Composition of the present invention (1% w/v)	SKJ-20-181-2
12	Composition of Gupta et al (1% w/v)	SKJ-20-187-6
18	Composition comprising (1%w/v) of *Emblica officinalis*	SKJ-20-187-4

15. The Figure attached as Exhibit 2 shows the qualitative TLC chromatogram. Spots 1, 7, and 13 are of extracts of the raw materials, namely *Sapindus trifoliatus*, and a 1:1 mixture of these two, respectively. The bands corresponding to sapinons are marked in the figure. *Emblica officinalis* does not show any band corresponding to the saponins, whereas *Sapindus trifoliatus* does show a band corresponding to saponins. The Figure is not intended to be a qualitative TLC chromatogram, and the intensities of the bands are not deemed to have relevance.

16. *In vivo* toxicology studies were performed in rats and dogs:

A. Rats: Antimigraine Formulation (3%) according to Gupta et al. patent, *Sapindus trifoliatus* (3%) and Mixture of *Sapindus trifoliatus* and *Emblica officinalis* (3%; 60:40) were studied for nasal irritancy. None of the formulations were found to be irritant to nasal mucosa, turbinate, bronchi and lungs.

B. Dogs: Antimigraine Formulation (1%) according to Gupta et al. patent, *Sapindus trifoliatus* (1%) and Mixture of *Sapindus trifoliatus* and *Emblica officinalis* (1%) were

studied for nasal irritancy. None of the formulations were found to be irritant to nasal mucosa, turbinate, bronchi and lungs.

17. Applicants have assayed saponins as hederagenin in the compositions as compared with the compositions of Gupta et al. The details are provided hereinbelow:

HPLC Assay of Saponins As Hederagenin In Anti-Migraine Nasal Preparation
Reagents used
Formic Acid (AR Grade)
Acetonitrile (HPLC Grade)
Water (Milli Q Grade)
Methanol (HPLC Grade)
Tetrahydrofuran (HPLC Grade)
Hydrochloric acid (AR Grade)

Preparation of Diluent
A mixture of THF and Methanol in the ratio 30:70 v/v was used as a diluent

Preparation of Standard Solution: About 50 mg of Hederagenin working standard was weighed and transferred to a 50 mL volumetric flask. 20 mL of diluent was added and sonicated to obtain a clear solution. The volume was made up using the diluent. 5mL of the standard solution was pipetted into a 50 mL volumetric flask and the volume was made up with methanol. The resulting solution was filtered through a 0.45 μ membrane filter. Estimation of Saponins as Hederagenin content in 1% Nasal Preparation of Gupta et. al. and present invention by acid hydrolysis. 15 mL of the samples were taken separately in a 100mL round bottom flask. 5ml of 50% methanolic hydrochloric acid was added and refluxed at 100 ^{0}C for 2 hours. The solutions were cooled and evaporated to dryness. 10 mL of diluent was added to each residue and sonicated for about 2 minutes. The contents were carefully transferred to 50 mL volumetric flasks and the volume was made up with methanol. The solutions were filtered through a 0.45 μm membrane filters. The above samples were analyzed in HPLC. The chromatographic conditions are as given below:

Instrumentation: A high performance liquid chromatograph system with gradient elution capability, autosampler with cooling chamber (Shimadzu class VP series) or equivalent.

Data handling system (Class VP, version 5.032 or equivalent): Analytical column: A stainless steel column 250 cm long, 4.6 mm in diameter filled with Octadecyl silica gel particle of 5 μm in diameter.(Used Kromasil C-18, 5μm (250 mm x 4.6 mm)

Preparation of Buffer: 1.0 mL of formic acid was added to 1000 mL volumetric flask and made up to the volume with water. The solution was filtered through a 0.45 or finer porosity membrane filter and degassed.

Chromatographic Parameters

Mobile Phase	:	25:75 (Buffer : Acetonitrile)
Flow rate	:	1 mL/min
Detector	:	UV at 205nm
Injection Volume	:	50 µL
Run time	:	20 min

Procedure: 50 µL of standard in triplicate and the specified sample solution in duplicate was injected, into the chromatograph. The chromatograms were recorded and the peak area of the main peak from the chromatographic report was measured. The retention time for Hederagenin was about ~ 7.75min.

Calculations

$$\text{Total saponins as Hederagenin (mg/mL)} = \frac{AT}{AS} \times \frac{DS}{DT} \times \frac{P}{100}$$

Where

AT = Average area counts of Hederagenin peak in the chromatogram of the sample solution

AS = Average area counts of Hederagenin peak in the chromatogram of the standard solution

DS = Dilution factor of the standard solution in mg/mL

DT = Dilution factor of the sample solution in mL/mL

P = Percent potency of Hederagenin working standard, on as is basis.

Details of Samples:

S. No.	Batch No	No of injections	Decoding	Figure Number
1	SKJ-22-183	3	Hederagenin Working STD (LLL3344)	Figure 1, 2, 3
2	NP-50-197	2	Formulation by Gupta et al Method	Figure 4, 5
3	LLL3344 Hederagenin	3	Hederagenin Working STD (SKJ-22-183)	Figure 6, 7, 8
4	MB-50-193	2	Formulation by Present invention Method	Figure 9, 10

18. The results obtained are tabulated in the following tables as given below (see also Exhibit 3).

TABLE A: Assay of 1% Anti-Migraine Nasal Preparation, Prepared By Gupta et al

SAMPLE NAME	Quantity taken	Makeup volume (mL)	Concentration	Area	Average area	Assay (mg /mL)

STD **STD POTENCY** **96.88** % w/w on as is basis	51.2 mg	500	**0.1024** (mg/mL)	2844979 2839659 2821331	**2835323** RSD 0.44%	
NP-50-197 (1% Formulation)	15 mL	50	**0.3** (mL/mL)	1219681 1220741	**1220211** RSD 0.06%	0.1423

TABLE B: Assay of 1% Anti-Migraine Nasal Preparation, Prepared By Present invention

SAMPLE NAME	Qtantity taken	Makeup volume (mL)	Conc.	Area	Average area	Assay (mg /mL)
STD **STD POTENCY** **96.88** % w/w on as is basis	47.1 mg	500	**0.0942**	**2697556** **2695518** **2693713**	**2695596** **RSD** **0.07%**	
MB-50-193 (1% Formulation)	15 mL	50	0.3	2868579 2878626	2873603 **RSD** **0.25%**	0.3243

19. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 of the laws of the United States, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

FURTHER DECLARANT SAYETH NOT

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